



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re application of: Court et al.

Application No. 10/692,553

Filed: October 23, 2003

Confirmation No. 1179

For: ENHANCED HOMOLOGOUS
RECOMBINATION MEDIATED BY
LAMBDA RECOMBINATION PROTEINS

Examiner: Jennifer Ann Dunston

Art Unit: 1636

Attorney Reference No. 4239-66898-01

CERTIFICATE OF MAILING

I hereby certify that this paper and the documents referred to as being attached or enclosed herewith are being deposited with the United States Postal Service as First Class Mail in an envelope addressed to: MAIL STOP AMENDMENT COMMISSIONER FOR PATENTS, P.O. BOX 1450, ALEXANDRIA, VA 22313-1450 on the date shown below.

Attorney or Agent
for Applicant(s)

Date Mailed

[Signature]
August 26, 2006

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DECLARATION UNDER 37 C.F.R. § 1.131

We, Neal Copeland, Daiguan Yu, Hilary M. Ellis, Donald E. Court, E-Chiang Lee, Nancy A. Jenkins, and Pentao Liu, declare as follows:

1. We are the inventors of the above-identified application, which is a continuation of U.S. Patent Application No. 10/366,044, filed February 12, 2003, which is a continuation-in-part of PCT Application No. PCT US01/25507, filed August 14, 2001, which claims the benefit of U.S. Provisional Application No. 60/225, 164, filed August 14, 2000 and claims the benefit of U.S. Provisional Application No. 60/271,632, filed February 21, 2001.

2. It is our understanding that the claims 1, 3, 4 and 13 are rejected as allegedly being anticipated by Cassanova et al., Genesis 32(2): 158-160, published online February 13, 2002.

3. We conceived of, and reduced to practice, a method for generating a vector for conditional knockout of a gene in a cell including a de-repressible promoter operably linked to a

nucleic acid encoding Beta and Exo, as claimed in claims 1, and 2-13, prior to February 13, 2002, in the United States.

3. The methods of claims 1, 3, 4 and 13 were conceived of prior to February 13, 2003. Selection cassettes for use in the claimed methods were made and improved prior to February 13, 2002; some of the experimental work conducted prior to February 13, 2002 is described below. Photocopies of Dr. Liu's laboratory notebook pages, labeled pages 1-10 are submitted herewith. The photocopied pages are referred to below as "the laboratory notes." Dates on these pages have been redacted. Prior to February 13, 2002, we performed the following experiments in the United States, which are documented on the laboratory notebook pages:

We constructed a plasmid that including a selectable marker (specifically a kanmycin/neomycin resistance marker) flanked by a pair of recombining sites (specifically LoxP). This plasmid was designed to introduce the recombining site into a genomic locus on a bacterial artificial chromosome (BAC) or a plasmid. A diagram of this plasmid, and a restriction map of this plasmid is shown in the laboratory notes, see page 1. The selection marker is called PL400.

We also constructed PL428 and PL430 which were additional plasmids for introducing recombining sites (LoxP sites) into the 5' and 3' sides of a genomic fragment of the Ctip2 locus. This is documented in the attached photocopy of Dr. Liu's laboratory notes, labeled page 2. DNA fragments of PL428 and PL430 were restriction digested or amplified by polymerase chain reaction. These fragments, containing the selectable marker (Kan-Neo) flanked by two recombining sites (LoxP) and having homology arms, were electroporated into E. Coli cells containing a de-repressible promoter (pL) operably linked to a nucleic acid encoding Beta and Exo. The production of kanamycin resistant cells is documented at the bottom of page 2 ("Kan^R"). A recombinase (Cre) is used to excise the nucleic acid encoding the selectable marker to leave a single first recombining site in the gene, as indicated on the right side of page 3 of the laboratory notes.

To clone a mouse genomic fragment from a BAC using recombineering, in order to make the conditional targeting vector, a retrieval vector (PL433) was constructed. PL433

includes two short DNA fragments from the end of the genomic DNA fragments. There is a MC1TK (thymidine kinase, a second selectable marker) in the backbone of this plasmid, negative selection could be used in embryonic stem cells with this conditional targeting vector. The production of PL433 is documented on page 4 of the laboratory notes.

The PL433 plasmid was electroporated into *E. coli* cells wherein the de-repressible promoter was de-repressed. Two colonies were examined by digesting the DNA with restriction enzymes. The restriction pattern documented that the selectable marker (TK) was inserted flanked by a second pair of recombining sites (LoxP). This produced plasmid PL435, shown on page 5 of the laboratory notes, which contained the genomic fragment (Ctip2) for making the targeting vector.

The DNA insert (2.8 kb in length) from PL430, which contained the selection marker (Kan-Neo) flanked by two recombining sites (loxP) was co-electroporated into bacterial (*E. Coli*) cells including a derepressible promoter (pL) operably linked to Gam and Exo. The cells were heat induced to insert the first recombining site into the Ctip2 locus. The correctly targeted plasmid was re-transformed into bacterial cells (*E. coli*). The loxP-flanked Kan marker was excised in the *E. coli* to leave a single loxP site in the genomic DNA. (see page 6 of the laboratory notes, top panel). This new plasmid was co-electroporated with the DNA fragment from PL436 containing the Neo-Kan selection marker also flanked by a second pair of LoxP sites. This resulted in the production of plasmid PL437. PL437 is the conditional knock-out vector that will allow deletion of the last exon of Ctip2 (see page 6 of the laboratory notes, bottom panel). The configuration of PL437 as a conditional targeting vector was confirmed using restriction digestion, as shown on page 7 of the laboratory notes.

A vector for conditional knock-out of the Evi9 locus was generated. This conditional targeting vector was designed to delete exon 4 of the Evi9 gene. The construction of this vector is shown on page 8 of the laboratory notes.

PL438 was a plasmid that contained a first pair of recombining sites (two LoxP sites, also called "floxed") flanking a selection marker (Neo-Kan), and flanked by two PCR amplified genomic DNA fragments. These genomic fragments could be used as homology arms in recombineering. The insert from this plasmid placed the floxed selection marker (Kan) into the 5' side of exon 4 (within exon 3) of the Evi9 gene. This plasmid could be used to introduce the first recombining sites into a BAC.

PL440 was a plasmid also contained a pair of recombining sites (LoxP or "floxed") flanking a selection marker (Neo-Kan) and flanked by a two polymerase chain reaction (PCR) amplified genomic DNA fragments. PL440 was of uses for recombineering. The insert from PL440 was used to place a floxed selection marker (Kan) into the 3' region of exon 4 (in intron 4) of the Evi9 gene. This plasmid could be used to introducing the second pair of recombining sites into a BAC.

PL441 was then constructed. This is a retrieval vector for retrieving the Evi9 genomic DNA fragment from an Evi9 BAC (see the bottom of page 8 of the laboratory notes). Linearized PL441 was electroporated into an Evi9 BAC (called "C3," see page 9 of the laboratory notes). The retrieved plasmid was called PL442. PL442 was co-electroporated with the insert from PL438 to place a floxed Neo-Kan selectable marker into intron 3 of Evi9 (see page 9 of the laboratory notes).

The targeted plasmid was transformed into *E. coli* expressing a recombinase ("Cre") to excise the selectable marker. This left a single LoxP site in intron 3 of Evi9. The production of this allele is shown in the top panel on page 10 of the laboratory notes.

The excised plasmid was then co-electroporated with the insert from PL440 to place a second floxed selectable marker (Neo-Kan) into intron 4 of Evi9. Thus, the plasmid PL443 was produced, which is a conditional targeting vector that could be used to delete exon 4 (located between intron 3 and intron 4) of Evi9. The production of PL443 is shown in the bottom panels on page 10 of the laboratory notes. We were aware that an Frt site could be used as a recombining site in the place of a loxP site, and that Flp could be used as the recombinase. A strain of *E. Coli*, EL250 was created that expresses Flp.

4. These results demonstrated: (1) homologous recombination could be used to insert a nucleic acid encoding a selectable marker (Neo-Kan) flanked by a pair of first recombining sites (LoxP) into a first site (one intron) in a gene (Evi9 or Ctip2) in vector including bacterial artificial chromosome (Evi9 or Ctip2), (2) homologous recombination could be used to insert a nucleic acid encoding a selectable marker (Neo-Kan) flanked by a pair of second recombining sites (LoxP) and a first recombining site into a second site (a second intron) in the gene (Evi9); (3) the nucleic acid encoding the selectable marker could be excised with a first recombinase specific (Cre) specific for the recombining sites, leaving a single first

recombining site in the gene (Evi9 or Ctip2), and (4) the nucleic acid encoding a selectable marker (Kan-Neo) could be excised with a recombinase (Cre) specific for the second recombining sites. Two recombining sites remained in the gene following excision of the nucleic acid encoding the selectable marker, thus generating a vector for conditional knockout of the gene (Evi9 or Ctip2). *E. coli* strains were created that expressed Flp, so that Frt recombining used. The homologous recombination was performed in bacterial cells including a de-repressible promoter (pL) operably linked to a nucleic encoding Beta and Exo.

5. All statements made herein and of our own knowledge are true and all statements made on information are believed to be true; and further, these statements were made with the knowledge that willful false statements and like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that any such willful false statements made may jeopardize the validity of the application or any patent issuing thereon.

Date _____

Neal G. Copeland

Date 7/26/2006

Daiguan Yu
Daiguan Yu

Date _____

Hilary M. Ellis

Date _____

Donald L. Court

Date _____

E-Chiang Lee

Date _____

Nancy A. Jenkins

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Pentao Liu

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PATENT TYPE

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AUG 23 2006

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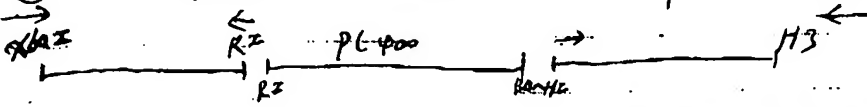
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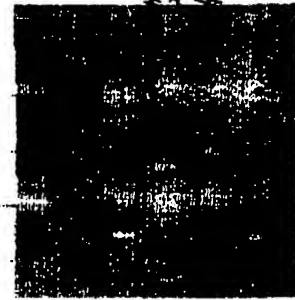
Pentao Liu

PCR fragments for making targeting vector to introduce 1st GxP.

① XbaI/RI XbaI-10966 Ctip2, RI-11274 Ctip2
 ② BsmHI/H3 BsmHI-11275 Ctip2, H3-11532 Ctip2



2λ	XbaI/RI	0
2λ	PG400	2
2λ	BsmHI/H3	0
2λ	PSK+	2
1λ	10x6ffu	1
1λ	T4 ligase	1
		4 the



1λ pA 2
 1λ dupp 2
 1λ prim 1
 1λ p-2
 1λ the 42

BEST AVAILABLE COPY

XbaI/XbaI - 2
 XbaI/H3 X-02

B-RI/H3 X546

①

digest PL428 #16 PL430 #2, #4 w/ NotI/SacI/PvuII
to excise the targeting cassettes.

15λ plasmid
4λ buffers
2λ enzymes (NotI, SacI, PvuII)

20λ H₂O

4λ

37°C

1.5 hr gel purify

spin column gel purify

QIAquick

20λ

use 3λ + 1λ PL424 (11002) #27

42°C → 240 kan^r

32°C → 4

electroporation Time constant 4

so can use more if needed

Can also amplify the whi- targeting vectors by PCR

① amplify PL428 w/ XbaI-13264-Ctip2

H3-13833-Ctip2

② PL430 w/ XbaI-10966-Ctip2

H3-1

use NotI/SacI/PvuII cut

PCR products were purified
by QIAquick → 28λ @ EB

PL428: 360ng/λ

PL430: 330ng/λ



1λ DNA
1λ dNTP
1λ primer
1λ primer
2λ H₂O
5λ 95°C 72°C
20λ

use 1λ PL424 #27 (11002)

plus 1λ PL430 330ng OR 2λ PL430 660ng

168 kan^r

176 kan^r

if use 32°C cells

0 kan^r

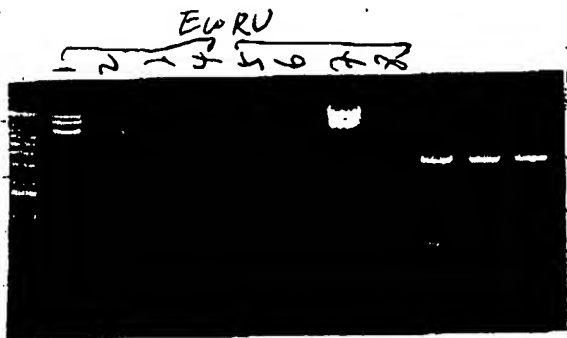
seems increased targeting DNA does not increase kan^r

might need more target DNA (PL424 #27)

mini prep

②

#1 → #4 from targeting w/ partial plasmid insert pL430
 #5 → #8 from - - - w/ PCR of pL430
 plasmid or ~~plasmid~~ insert from plasmid is better.



use #2 electrop → E6350 → Kan^r
 #2 → CRE induced E6350 → Kan^r Amp^r
 #1, #2 from
 #3, #4 - still mixed
 after pop out



use #4 mixed-popout cells directly
 and electroporated w/ pL430 partial cassette
 CRE induced. no Kan colony.
 indicating co-electroporation is a must

- primers
- ① NotI-clp2-ret-5'-1-4001
H3-clp2-ret-5'-2-4371
 - ② H3-clp2-ret-3'-1-17779
speI-clp2-ret-3'-2-18145

- ③ BamHI-CG9650-5' }
④ XhoI-CG9650-3' }
⑤ Same as ③

- ⑥ Same as ⑤

#1, #2 work
#3 → #6 do not work

#1, #2 were purified to QAgarose and digested
w appropriate enzymes, and again purified
w QAg.

11-24-01.

#1 cut w NotI/H3, #2 cut w H3/speI, pL433, NotI/speI

ligation

- 3λ #1
- 3λ #2
- 2λ pL433
- 1λ 10x buffer
- 1λ Taq ligase

DNA
CS

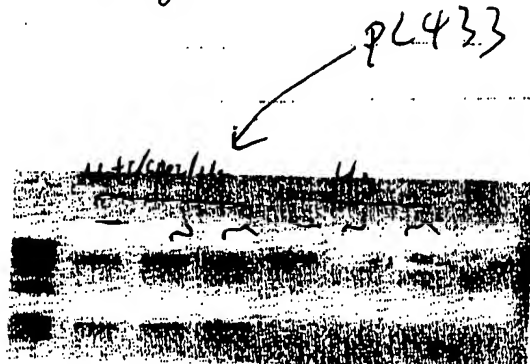
Fly adult RT. Random priming

Fly adult RT. oligo dT priming

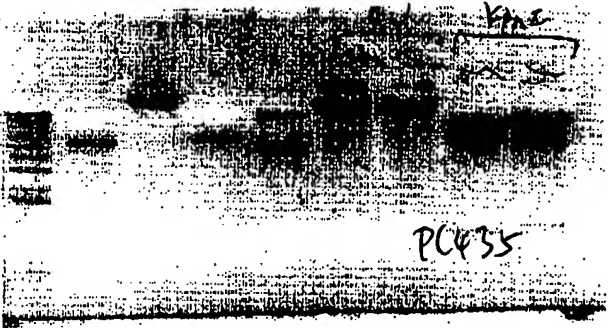
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- 1X DNA
- 1X dNTP
- 1X primer1
- 1X primer2
- 21X H₂O
- 5λ 10x
- 0.75λ Tag
- 20λ H₂O



5PL433 (1309/12) 42°C 1A
 42°C induced CT92: C5 BAC
 Amp plate.



12-11-19

9AM. inoculate EL350
 PL435 co-electroporation in PL430 insert to target the 1st LxP to the cfpz retrieved plasmid.

42°C 120 colonies, 32°C 10 colonies.

pick up ~~four~~ four, 2 are correlated, #3, #4.

no mixture of targeted plasmid

#4 dilute 100x
 → EL350 (core) to pop out
 → DH10B
 BamHI 4.5 kb 19 kb
 7.5 kb
 RV 11 kb 10 kb 10 kb 9 kb



9AM. inoculate EL350 directly from pop out colonies. EL350 (pop out) → 100x plate 200-300 colonies. DH10B thousands need dilution in 100µl of Amp.
 inoculate two → grow 10 hrs → miniprep
 one has 50ng/µl, and 110ng/µl DNA
 use the mid one 1λ + 1λ PL436 cassette (150ng/λ)
 ↓
 42°C inoculated EL350

42°C 102 colonies

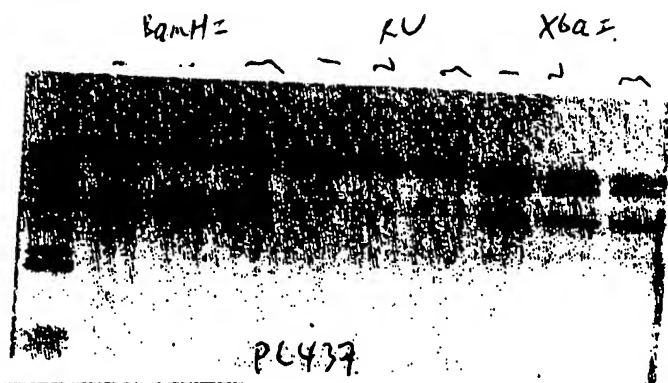
32°C 9 colonies, pick up 4 → 32°C → o/v



#2, 3, 4 are mixed w/ targeted and non-targeted take 1µg of #4 → DH10B

PL437

miniprep 3 columns of DH10B



cfp
Conditional
target
Vector



EVG4 exon 4 retrig & 3' LoxP PCR

primers

DNA

1. MetI-EVig-exon4-retrve 5'-1
H3-EVig-exon4-retrve 5'-2

J8AC C3
A5

1λ DNA 4
1λ WTP 4
1λ prime 1
1λ prime 2
21λ H2O 84

2. H3-EVig-exon4-retr 3'-1
H3-EVig-exon4-retr 3'-2

Same

3. XbaI-EVig-exon4-3'-L1
RI-EVig-exon4-3'-L2

Same

4. BamHI-EVig-exon4-3'-K1
H3-EVig-exon4-3'-R2

Same

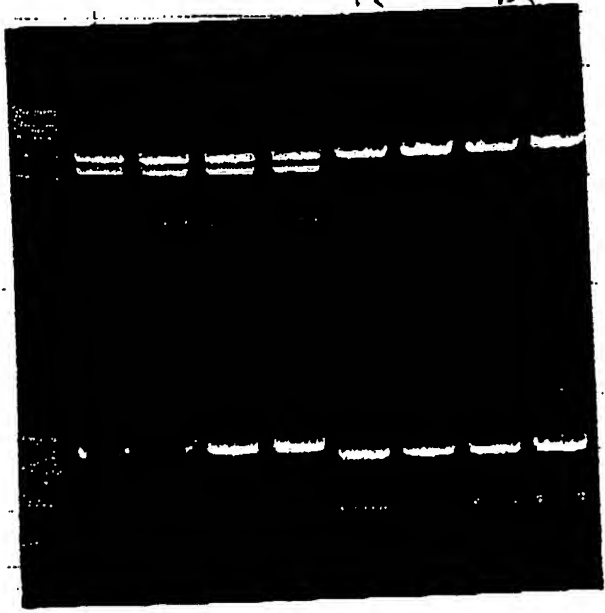
5λ 2 20
0.75λ 1 3
20λ 80



cut purify w columns and cut. → purify

1. 3λ
2. 3λ
PCR 100/100 2λ
Eox 1λ
TE 1λ

3. 20λ
4. 20λ
PCR 100/100 3λ
PCR 100/100 2λ
Eox 1λ
TE 1λ



PL440: LoxP to 3' of EVig, exon4

PL441: retrig vector for exon4, EVig

E13 BAC A5. C3 electroporated to EL350 $\approx 300-400$ colonies
 electroporated H3 at PL441 \rightarrow A5. C2 BACS
 thousands ~~amp~~ colonies recovered
 pick 4 2 (#1, #2) from A5, 2 (#3, #4) from C3
 10 hours at 32°C w/ Amp. miniprep.

only #4 (maybe #3) is correct, indicate A5 BAC may not have any
 take 2 λ #4, \rightarrow 200 λ TE, take 1 $\lambda \rightarrow$ DH10B (7 λ) in 60
 plate 100 λ , 10³ colonies grow up. pick

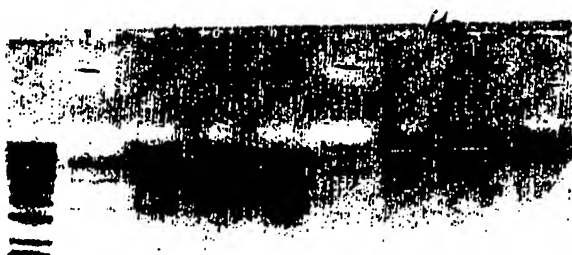
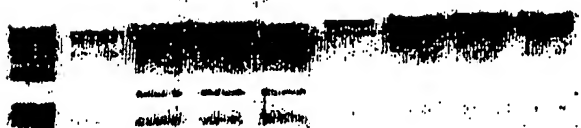
#1 - #2. original #4 prep

#3 - #4. original #2 prep (in-correct)



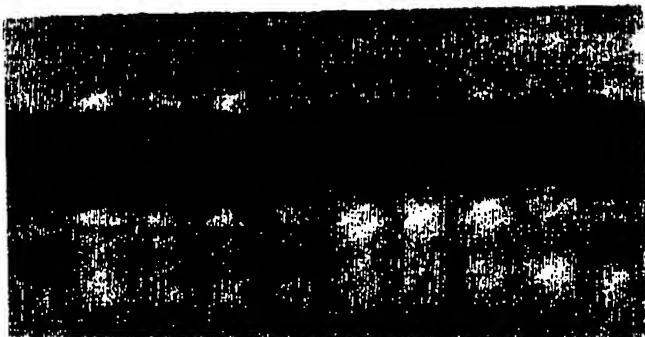
PL438 #1, \rightarrow long λ 3 λ each electroporate \rightarrow EL350 etc.
 PL442 #2 \rightarrow long λ 0.5 λ each
 plate on wrong antibiotics (cm), re-do.

re purify PL438 #1, 500 λ . use 2 λ , use 75 ng PL442 #2 \rightarrow EL350
 incubate, 24-32°C 20' at 32°C incubator (waitly for water bath to rise). 42°C \rightarrow 5000 Kan. plate up 4.



all are targeted.
 but #4 also not have WT confirmation
 \rightarrow used for pop out.

EVIS exon4 conditional allele Est Corp pop out



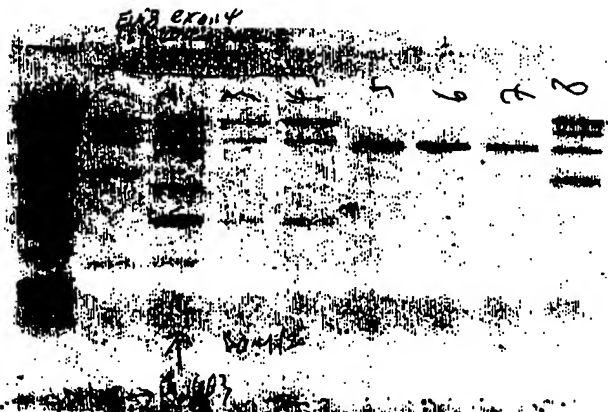
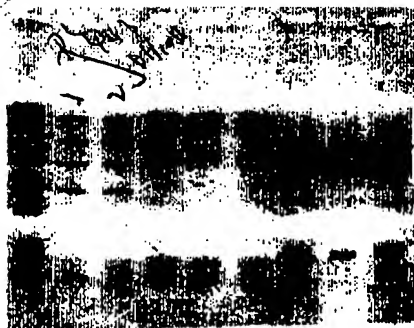
1-1-02

targeting the pop out plasmid #2 ^{100ng} pL440 insert into
using the frozen EL350 competent cells $\rightarrow \sim 4 \times 10^8$ colonies
3 targeted. Transform #2 \rightarrow DHAPB Kanamycin plate.

2nd 10x PCR
in 100ul
100ul

pick up 4 colonies.

pL443



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